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SNAP-25a and -25b isoforms are both expressed in insulin-secreting cells and can function in insulin secretion

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The tSNARE (the target-membrane soluble NSF-attachment protein receptor, where NSF is *N*-ethylmaleimide-sensitive fusion protein) synaptosomal-associated protein of 25 kDa (SNAP-25) is expressed in pancreatic B-cells and its cleavage by botulinum neurotoxin E (BoNT/E) abolishes stimulated secretion of insulin. In the nervous system, two SNAP-25 isoforms (a and b) have been described that are produced by alternative splicing. Here it is shown, using reverse transcriptase PCR, that messages for both SNAP-25 isoforms are expressed in primary pancreatic B and non-B cells as well as in insulin-secreting cell lines. After transfection, both isoforms can be detected at the plasma membrane as well as in an intracellular perinuclear region in the insulin-secreting cell line, HIT. To test for the functional role of the two isoforms in insulin secretion, mutant forms of SNAP-25a and b resistant against cleavage by BoNT/E were generated. Such mutant SNAP-25, when expressed in HIT cells, is not inactivated by BoNT/E and its ability to restore insulin secretion can thus be investigated. To obtain the toxin-resistant mutant isoforms, the sequence around the BoNT/E cleavage site (R^{176}QIDRIM^{182}) was changed to P^{176}QIKRIT^{182}. This is the sequence of the equivalent region of human SNAP-23 (P^{187}–T^{194}), which has been shown to be resistant to BoNT/E. The mutant SNAP-25 was resistant to BoNT/E *in vitro* and *in vivo* and both mutant isoforms were able to reconstitute insulin secretion from toxin-treated HIT cells.

Key words: exocytosis, islet, neurotoxin, regulated release, SNARE.

INTRODUCTION

Pancreatic B cells store insulin in large dense-core granules. When blood glucose levels rise they are stimulated to release stored insulin by exocytosis [1,2]. The release mechanism of insulin from large dense-core granules has been shown to be very similar to the secretion mechanism of neurotransmitters at the synapse. Most of the molecular components of the secretion machinery described in neuronal cells have also been shown to be present in pancreatic B-cells. Thus the ATPase NSF (*N*-ethylmaleimide-sensitive fusion protein) and the soluble NSF-attachment protein (SNAP), which are essential for neurotransmitter release, are involved in insulin secretion [3]. In addition, VAMP-2 (vesicle-associated membrane protein 2), the vesicle-membrane SNAP receptor (vSNARE) present on synaptic vesicles, has been detected on insulin-containing granules [4]. Tetanus toxin cleavage of VAMP-2 impairs insulin secretion, indicating that, as in neurons, VAMP-2 is essential for regulated release in endocrine cells [4,5]. Two target-membrane SNAP receptors (tSNAREs), syntaxin 1 and SNAP-25 (synaptosomal-associated protein of 25 kDa), which have been studied extensively in neuronal cells, are also found in pancreatic B cells and both have been shown to be implicated in insulin release using different assay systems [6–10].

In neurons and other cell types, multiple isoforms of vSNAREs and tSNAREs have been described and are thought to be involved in different cellular-fusion events [11]. The VAMP family consists of three different isoforms; VAMP-1 and -2, expressed mainly but not exclusively in neuronal and neuroendocrine cells [12]; and cellubrevin, a more widely expressed isoform [13]. The syntaxin family of tSNAREs is composed f more than 12 isoforms with different tissue distributions as well as different cellular localizations. Whereas syntaxin 1a and 1b are essentially neuron (or neuroendocrine) specific, syntaxins 2–6 display a broader tissue distribution [14]. Syntaxins 1–4 are plasma-membrane-associated [14–16], syntaxins 5, 6, 10 and 16 localize to the Golgi region [14,17–20] and syntaxins 7 and 12 to endosomes [21–23].

In neurons, two SNAP-25 isoforms (a and b) have been described [24,25] and more recently a ubiquitously expressed isoform called SNAP-23 has been cloned [26]. The two neuronal isoforms arise from alternative splicing of two divergent versions of exon 5 [24,25]. They differ by only nine amino acids and notably in the spacing of four cysteine residues, which are palmitoylated and participate in the membrane association of SNAP-25 [27–29]. The expression level of the two isoforms is developmentally regulated in the nervous system. SNAP-25a is more abundant during early development, and SNAP-25b is the predominant isoform in the adult [30]. This, however, does not apply to all brain regions. Even in the adult in some brain regions a reciprocal expression pattern of messages for the two isoforms has been described [31]. Likewise it has been shown that SNAP-

Abbreviations used: SNAP-25, synaptosomal-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF-attachment protein; tSNARE, target-membrane SNAP receptor; vSNARE, vesicle-membrane SNAP receptor; SLO, streptolysin-O; BoNT/E, botulinum neurotoxin E; RT, reverse transcriptase; DPBS, Dulbecco's PBS.

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25a and not b is the main isoform in adult adrenal and pituitary cells [30].

So far it is not known which isoform is expressed in insulin-secreting cells and what the functional difference is between the two isoforms. Here we show that messages for both isoforms are found in insulin-secreting cells and that there are no apparent differences in the cellular localization of SNAP-25a and b. Furthermore, both isoforms are able to function in regulated insulin release.

EXPERIMENTAL

Materials

The cDNA coding for SNAP-25a and b [25] and the C-terminal *myc*-tagged constructs (for details, see [30]) were obtained from Dr. M. C. Wilson (Scripps Research Institute, La Jolla, CA, U.S.A.). For the N-terminal *myc*-tagged SNAP-25 constructs the *myc* epitope was placed into the *Nco*I side of the SNAP-25 cDNAs. The different SNAP-25 constructs and the CD4 cDNA were cloned in pcDNA3 and the human proinsulin in pCMV. In all experiments using botulinum neurotoxin E, only the light chain of the toxin (prepared as a recombinant protein; T. Binz, T. Hayashi, S. Yamasaki, and H. Niemann, unpublished work) was used; and referred to below as BoNT/E. Recombinant steptolysin-O (SLO; with Cys^{530} mutated to Ala) fused to the *Escherichia coli* maltose-binding protein was used for cell permeabilization [32]. The monoclonal anti-SNAP-25 antibody was purchased from Sternberger Monoclonals Inc. (Baltimore, MD, U.S.A.) and the secondary antibody, anti-mouse FITC, was from Antibodies Inc. (Davis, CA, U.S.A.).

Isolation of primary pancreatic islet cells

Pure islet B and non-B cells were obtained as described previously [33]. In brief, pancreata from six rats were digested with collagenase in Ca^{2+}-containing Hanks buffer, and islets of Langerhans were separated from exocrine tissue by discontinuous density-gradient centrifugation (Histopaque 1077 from Sigma). Islets were treated with trypsin and B cells were sorted from non-B cells by size and FAD auto fluorescence using a fluorescence-activated cell sorter [33]. This method has been shown to yield one population consisting of 95% B cells, and a second with 93% non-B cells, as shown by classical double-immunofluorescence techniques [33].

Cell lines

RIN cells (rat) [34] were cultured in RPMI 1640/10% fetal-calf serum/2.05 mM glutamine and HIT cells (hamster) [35] were cultured in RPMI 1640/10% fetal-calf serum/2.05 mM glutamine/32.5 μM glutathione/0.1 μM seleneous acid.

Estimation of SNAP-25a and b mRNA levels

Total RNA was isolated from primary pancreatic islet B and non-B cells as well as from the insulin-secreting cell lines HIT and RIN using trizol (Life Technologies Inc.) according to the manufacturer's instructions. RT (reverse transcriptase)-PCR was performed using 5′-AAGATGCTGGCATCAGGACT-3′ as upstream primer located in SNAP-25 exon 4 and 5′-CTGCGGATGAAGCCACCACT-3′ as downstream primer located in exon 6. The downstream primer was phosphorylated with [γ-^{32}P]ATP. The amplified SNAP-25 fragment was purified from an agarose gel and digested with either *Nde*I (cleaving in exon 5a) or *Sty*I (cleaving in exon 5b) or both. The digested fragments were electrophoresed on a non-denaturing 8% polyacrylamide gel, which was dried and exposed for autoradiography.

Transfection of HIT cells

HIT cells were cultured at a density of 4.5×10^5 cells/well either on plastic 24-well plates for secretion assays or on glass coverslips (placed in 24-well plates) for immunofluorescence. Transfections were performed using 1 μl of Transfectam (Promega) and 5 μg of total DNA per well in a total volume of 250 μl of culture medium without fetal calf serum for 6 h in the incubator. In order to obtain similar expression levels of the SNAP-25 isoforms, five times more DNA of the SNAP-25a construct was used. In detail: for secretion assays, isoform SNAP-25a, 2.5 μg of pCMV-human proinsulin/0.5 μg of pcDNA3-SNAP-25a mutant/2 μg of pcDNA3 (empty vector); for secretion assays, isoform SNAP-25b, 2.5 μg of pCMV-human proinsulin/0.1 μg of pcDNA3-SNAP-25b mutant/2.4 μg of pcDNA3; for sorting, isoform SNAP-25a, 2.5 μg of CD4 plasmid/2.5 μg of pcDNA3-SNAP-25a mutant; for sorting, isoform SNAP-25b, 2.5 μg of CD4 plasmid/0.5 μg of pcDNA3-SNAP-25b mutant/2 μg of pcDNA3; for immunofluorescence, all constructs, 2.5 μg of *myc*-tagged constructs/2.5 μg of pcDNA3.

Immunofluorescence

After transfection (2 days), cells were fixed for 10 min in 4% paraformaldehyde in PBS, washed twice in Dulbecco's PBS (DPBS), permeabilized for 30 s in acetone, washed twice with DPBS and blocked for 15 min in DPBS plus 5% BSA before incubation with the first antibody, anti-Myc (hybridoma supernatant diluted 1:10) or monoclonal anti-SNAP-25 (Sternberger Monoclonals Inc., diluted 1:400), for 2 h at room temperature. Cells were then washed twice in DPBS and incubated with the secondary antibody, anti-mouse FITC, diluted 1:1000 for 1 h at room temperature in the dark. Finally, cells were washed twice in DPBS and mounted using Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.). Fluorescence images were taken using a Cool View camera adapted to a fluorescence microscope (Zeiss).

Mutagenesis of SNAP-25 isoforms

SNAP-25a and b cDNAs cloned in the expression vector pcDNA3 were mutated using the QuickChange®Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions, using 5′-CCCAGAATCCCCAGATTAAGAGGATCACGGAGAAGGC-3′ and 5′-GCCTTCTCCGTGATCCTCTTAATCTGGGGATTCTGGG-3′ as primers.

Incubation of *in vitro*-translated SNAP-25 proteins with BoNT/E

In vitro transcription/translation of SNAP-25 wild-type and mutant isoforms was performed in presence of [^{35}S]methionine (NEN DuPont) according to the protocol supplied by the manufacturer of the translation kit (Promega). The *in vitro*-translated protein (1 μl) was incubated in a total volume of 100 μl of low-Ca^{2+} glutamate buffer (as used in the secretion assay, described below) with BoNT/E at a final concentration of 10 or 50 nM for 10 min or 3 h at 37 °C. Samples were then boiled in SDS sample buffer and subjected to SDS/PAGE and autoradiography.

Secretion–reconstitution assay

HIT cells were washed twice with Krebs–Ringer buffer lacking Ca^{2+} (125 mM NaCl/5 mM KCl/2 mM $MgSO_4$/1.2 mM

KH_2PO_4/25 mM Hepes/5 mM glucose/0.4 mM EGTA, pH 7.4) and then permeabilized with 9 μg/ml SLO for 7 min at 37 °C in low-Ca^{2+} glutamate buffer (128 mM K^+-glutamate/5 mM NaCl/7 mM $MgSO_4$/20 mM Hepes/5 mM Na_2ATP/0.5 mM $CaCl_2$/10.2 mM EGTA, pH 7; corresponding to 0.1 μM free Ca^{2+}). Permeabilized cells were incubated in the presence or absence of 30 nM BoNT/E in low-Ca^{2+} glutamate buffer for 8 min at 37 °C. Buffer was then replaced with either low-Ca^{2+} glutamate buffer or high-Ca^{2+} glutamate buffer (128 mM K^+-glutamate/5 mM NaCl/7 mM $MgSO_4$/20 mM Hepes/5 mM Na_2ATP/9.8 mM $CaCl_2$/10.2 mM EGTA, pH 7; corresponding to 10 μM free Ca^{2+}) for a secretion period of 7 min at 37 °C. Secretion from transfected cells was estimated by measuring the amount of human C-peptide released into the medium using ELISA (Dako, Cambridge, U.K.). This ELISA does not cross-react with the endogenous hamster C-peptide of the HIT cells.

Sorting of CD4-expressing cells

After co-transfection of CD4 and SNAP-25 mutants (2 days), cells were trypsinized. The cell suspension (2×10^7 cells in 18 ml of complete medium) was incubated with an anti-CD4 monoclonal antibody coupled to magnetic beads (1.3×10^7 beads; Dynal, Oslo, Norway) for 1 h at 4 °C with gentle agitation. Transfected cells were then separated from untransfected cells using a magnetic-tube holder. Positive cells attached to the magnetic beads were washed twice with medium and plated at a density of 1.5×10^5 cells/well into 96-well plates coated with an extracellular matrix secreted by 804G cells [36]. Culture was continued for 1 day before cells were used for toxin treatment, identical with that used for the secretion assay, and finally lysed in SDS sample buffer for SDS/PAGE.

SDS/PAGE and Western blotting

Proteins were separated on 10% polyacrylamide gels under reducing or non-reducing conditions, as indicated in the Figure legends, according to Laemmli [37]. After SDS/PAGE, proteins were transferred on to nitrocellulose (Schleicher & Schuell) and SNAP-25 was detected using the monoclonal antibody, SMI 81, at a dilution of 1:1000, an anti-mouse horseradish-peroxidase-conjugated second antibody and the enhanced chemiluminescence detection system (Amersham).

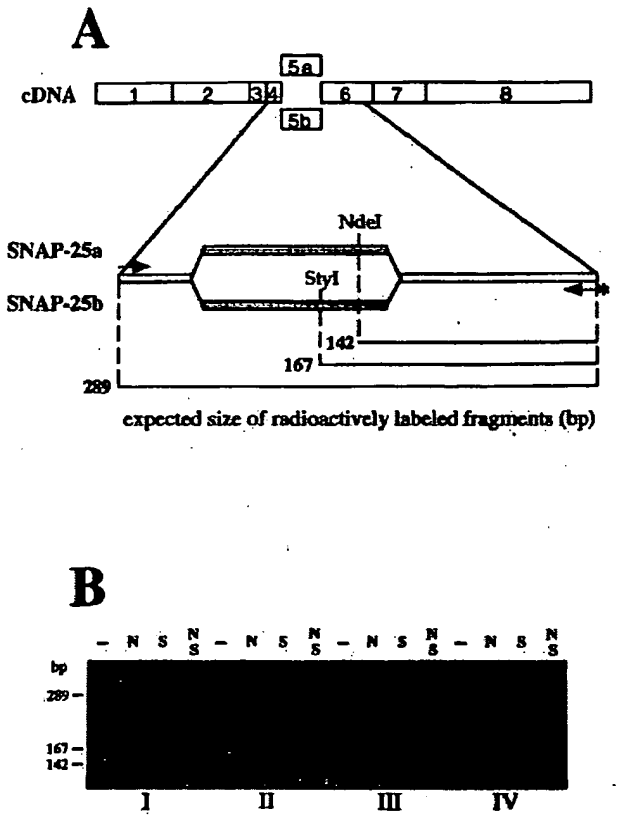

Figure 1 Expression of messages for SNAP-25a and b isoforms in insulin-secreting cells

(A) Schematic representation of the SNAP-25 fragments obtained by RT-PCR and the cleavage sites of the restriction enzymes *NdeI* and *StyI*, located in exons 5a and 5b respectively. (B) RT-PCR using HIT (I on Figure), RIN (II), rat islet non-B (III) or B (IV) cell RNA for amplification of SNAP-25 exons 5a and 5b in the presence of a radioactively labelled downstream primer. The amplified DNA fragment was incubated with the restriction enzyme *NdeI* (which cleaves only in exon 5a, giving rise to a 142 bp fragment, lanes N), *StyI* (which cleaves only in exon 5b, giving rise to a 167 bp fragment, lanes S), with both of them (lanes N/S), or without any restriction enzyme (lanes –).

RESULTS

Detection of mRNAs for SNAP-25 isoforms in insulin-secreting cells

So far, no antibody is available to distinguish between the two SNAP-25 isoforms. In order to determine whether one or both isoforms are expressed in insulin-secreting cells, we tested for relative mRNA levels of the two isoforms. Total RNA was isolated from primary rat pancreatic B and non-B cells as well as from the two insulin-secreting cell lines HIT (hamster) and RIN (rat). To distinguish between RNA levels for SNAP-25a and b, a SNAP-25 fragment containing the alternatively spliced exon 5 was amplified by PCR from reverse-transcribed RNA. This PCR fragment was then digested with the restriction enzymes *NdeI* or *StyI*, cleaving only in exons 5a or 5b, respectively (see schematic illustration in Figure 1A). These restriction enzymes have been used by Jagadish et al. to identify isoform messages in insulin-responsive tissues [38]. As shown in Figure 1(B), all four RNA preparations contain messages for both SNAP-25 isoforms. In primary cells the SNAP-25a message seems to be more abundant, whereas in RIN cells the amounts of message for SNAP-25a and b isoforms are similar. A considerable amount of the SNAP-25 fragment amplified from HIT-cell RNA was not cleaved in the double digest (Figure 1B, section I, N/S). This may be due to heteroduplex formation between the two hamster isoform DNAs or eventually to the presence of an additional isoform. In any case, the intention of this experiment was to answer the question of whether both isoforms are expressed in insulin-secreting cells, and the results show unequivocally that this is the case.

Cellular localization of SNAP-25 isoforms in HIT cells

We have shown previously that endogenous SNAP-25 is localized at the plasma membrane as well as in a perinuclear region [39]. Given the fact that mRNA for both SNAP-25 isoforms is detected in insulin-secreting cells and that there are predicted differences in their palmitoylation patterns, the question arises as to whether the two isoforms differ in their localization with respect to the plasma membrane or perinuclear region. To address this issue, cDNAs encoding SNAP-25a and b tagged with the *myc*-epitope were prepared. The epitope was placed at either the N- or the C-terminal sides to minimize artifacts due

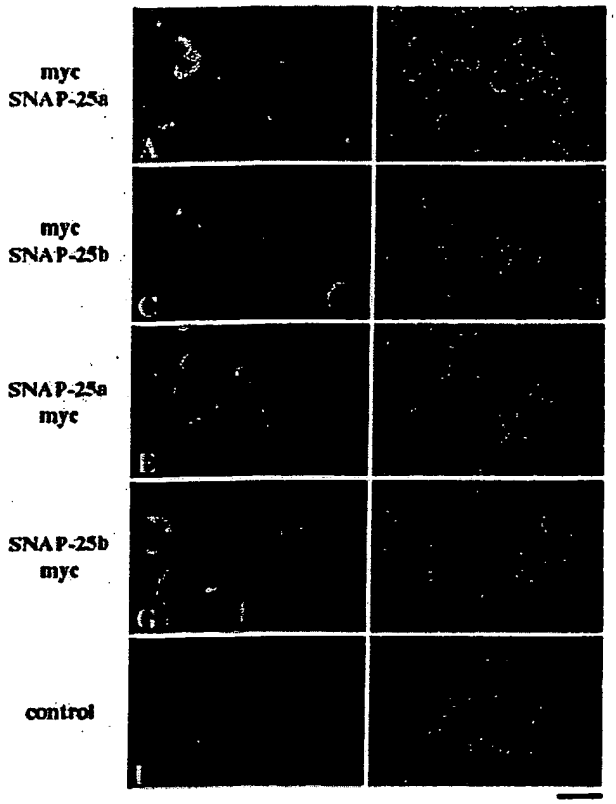

Figure 2 Immunolocalization of Myc-tagged SNAP-25 isoforms in HIT cells

HIT cells were transfected with: SNAP-25a Myc-tagged at the N-terminus (**A**, **B**); SNAP-25b Myc-tagged at the N-terminus (**C**, **D**); SNAP-25a Myc-tagged at the C-terminus (**E**, **F**); SNAP-25b Myc-tagged at the C-terminus (**G**, **H**) or empty expression vector (**I**, **J**). After transfection (2 days) cells were fixed and incubated with a monoclonal antibody against the Myc epitope (**A**–**H**) or against SNAP-25 to stain for endogenous SNAP-25 as a control (**I**, **J**). Fluorescence, **A**, **C**, **E**, **G** and **I**; phase contrast, **B**, **D**, **F**, **H** and **J**. Scale bar, 20 μm; same magnification for all images.

to any impact of the Myc-tag on intracellular targeting. HIT cells were transfected with these constructs and 2 days later immunostained with an antibody against Myc. As shown in Figure 2, there was no apparent difference between the localization of either the Myc-tagged SNAP-25 isoforms or the exogenously and the endogenously expressed SNAP-25. SNAP-25 from all constructs as well as the endogenous SNAP-25 was located mainly at the plasma membrane and, in addition, in some of the cells, in a perinuclear region.

Production of BoNT/E-resistant SNAP-25 isoforms

We have shown previously that SNAP-25 is implicated in the release mechanism of insulin, using BoNT/E as a tool [39]. BoNT/E introduced into permeabilized HIT cells cleaves SNAP-25 and Ca^{2+}-induced insulin secretion is strongly inhibited. In order to test for the functional activity of SNAP-25 isoforms in the process of insulin secretion, we produced mutants of the two isoforms resistant against toxin cleavage. Such isoforms can then be expressed in HIT cells in which the endogenous SNAP-25 will be inactivated by the toxin. To this end, we mutated in both isoforms amino acids Arg^{176}, Asp^{179} and Met^{182} to Pro^{176}, Lys^{179} and Thr^{182}, respectively, resulting in the sequence $P^{176}QIKRIT^{182}$ rather than $R^{176}QIDRIM^{182}$. This corresponds to the equivalent

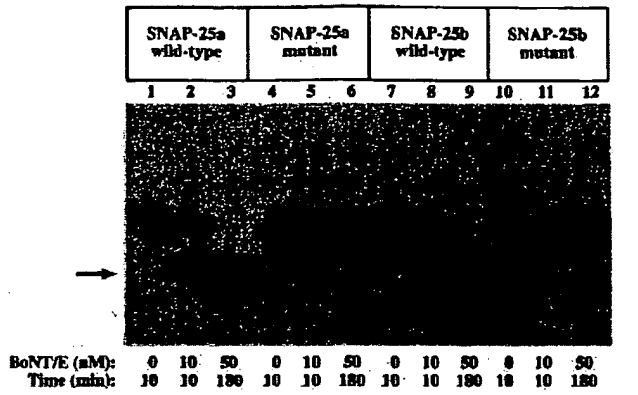

Figure 3 Mutant SNAP-25a and b isoforms are resistant to cleavage by BoNT/E

In vitro-translated, radioactively labelled, SNAP-25a (lanes 1–6) and SNAP-25b (lanes 7–12), wild type (lanes 1–3, 7–9) and mutant (lanes 4–6, 10–12) forms, incubated in the presence (10 or 50 nM as shown) or absence of BoNT/E at 37 °C for the times indicated. SDS/PAGE was performed under reducing conditions. Cleavage by the toxin lead to the appearance of a new radioactive band of smaller molecular mass, as indicated by the arrow.

sequence in (human) SNAP-23 (a ubiquitously expressed SNAP-25 analogue), which we have shown previously to be resistant against cleavage with BoNT/E [40]. Radioactively labelled mutant or wild-type SNAP-25 isoforms obtained by *in vitro* translation were incubated with BoNT/E. Figure 3 shows that the mutant SNAP-25 isoforms are completely resistant against BoNT/E cleavage, even after a 3 h incubation with 50 nM BoNT/E, whereas most of the wild-type SNAP-25 was already cleaved after 10 min with 10 nM BoNT/E.

Functional role of SNAP-25 isoforms in the process of insulin secretion

To test for the functional activity of the mutant SNAP-25 isoforms, HIT cells were co-transfected with human insulin (as a reporter for the amount of secretion uniquely from the subpopulation of transfected cells [41]) and the SNAP-25a or b mutants, or the empty vector as a control. After transfection (2 days) the cells were permeabilized and treated with 30 nM BoNT/E to inactivate the endogenous SNAP-25. Insulin secretion was then induced by raising the Ca^{2+} concentration in the incubation medium. Figure 4(A) shows that both mutant isoforms are able to replace the endogenous SNAP-25 in the process of insulin secretion. By contrast, transfection of the cells with wild-type SNAP-25 isoforms did not rescue the Ca^{2+}-induced insulin secretion, in keeping with its cleavage by the toxin even following its over-expression (results not shown and [40]). The amount of DNA used for transfection of the toxin-resistant mutants was adjusted to obtain a reasonable and comparable expression level for the two isoforms, as judged by Western blots of total cell lysates after the secretion assay (Figure 4B). Assuming that approximately 10% of the cells were transfected in our transient transfections, the expression level of the SNAP-25 mutants was comparable with that of the endogenous SNAP-25.

To rule out the possibility that the presence of toxin-resistant SNAP-25 mutants might prevent toxin cleavage of the endogenous SNAP-25, the mutants were this time highly over-expressed in cells co-transfected with CD4. After 2 days, cells were treated with trypsin and transfected cells were then sorted from untransfected cells using anti-CD4 antibodies coupled to

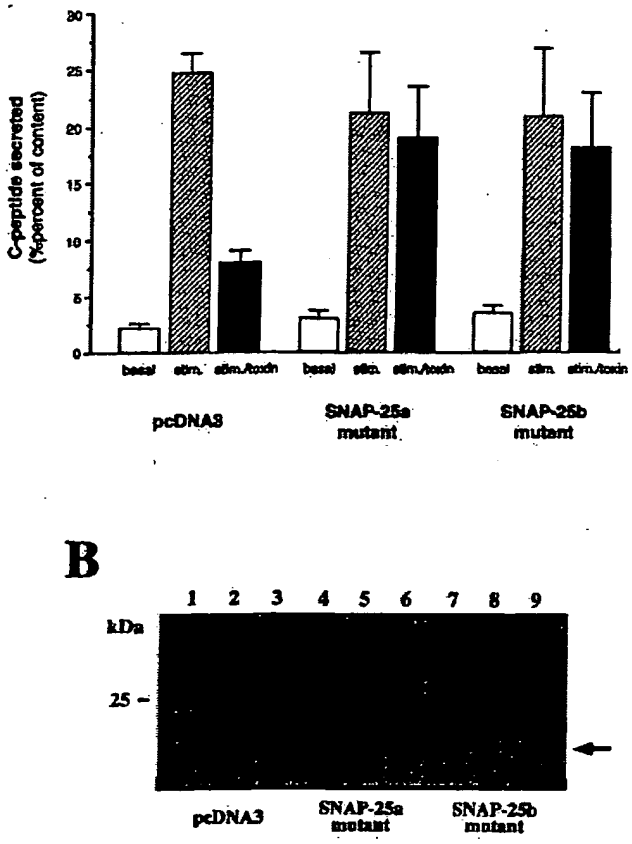

Figure 4 SNAP-25a and b are both able to support regulated insulin secretion

(A) HIT cells were transiently co-transfected with human proinsulin and the empty vector or one of the toxin-resistant SNAP-25 isoforms. After transfection (2 days) cells were permeabilized with SLO and incubated with (/toxin) or without 30 nM BoNT/E for 8 min. The medium was then replaced by a high (stim.)- or low (basal)-Ca^{2+}-containing buffer for a secretion period of 7 min. Insulin release from transfected cells was estimated by measuring the amount of human C-peptide released into the medium (results are expressed as percentage of content, values are means ± S.E.M., $n = 4$). (B) At the end of the secretion assay described in (A), cells were lysed in SDS sample buffer (under reducing conditions) and total cellular proteins were separated by SDS/PAGE (a quarter of a well was loaded/lane). Lanes 1, 4 and 7, basal release condition; lanes 2, 5 and 8, Ca^{2+}-stimulated conditions; lanes 3, 6 and 9, Ca^{2+}-stimulated conditions after BoNT/E treatment. Proteins were transferred to nitrocellulose and incubated with the monoclonal antibody against SNAP-25. Cleavage of endogenous (but not mutant) SNAP-25 lead to the appearance of a new immunoreactive band of smaller molecular mass, as indicated by the arrow (for unknown reasons the signal of the cleaved SNAP-25 protein was usually less intense than the signal for the intact protein, especially under reducing conditions). The faint bands at 25 kDa in lanes 6 and 9 correspond to the mutant isoforms resistant against toxin cleavage.

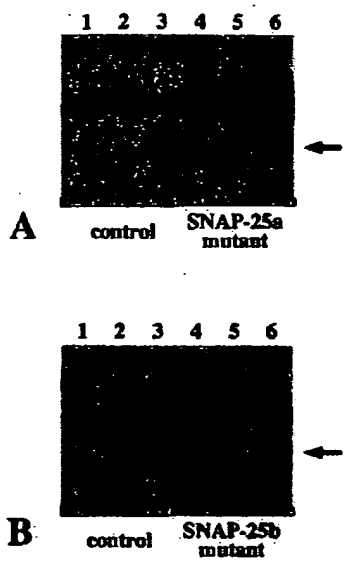

Figure 5 Overexpression of toxin-resistant (mutant) SNAP-25a or b does not prevent cleavage of endogenous (native) SNAP-25 by BoNT/E

HIT cells were co-transfected with CD4 and either the empty vector or BoNT/E-resistant SNAP-25a or b mutants (A and B, respectively). Transfected cells were sorted 2 days later from untransfected cells with anti-CD4 antibodies coupled to magnetic beads. Culture of sorted cells was continued for 1 day and cells were finally permeabilized and treated with toxin as described in the legend to Figure 4. Thereafter cells were lysed in SDS sample buffer, and total cellular proteins were separated by SDS/PAGE under non-reducing conditions [one well of a 96-well plate was loaded/lane in (A) and half of a well/lane in (B)]. Lanes 1 and 4, basal release conditions; lanes 2 and 5, Ca^{2+}-stimulated conditions; lanes 3 and 6, Ca^{2+}-stimulated conditions after BoNT/E treatment. Proteins were transferred to nitrocellulose and incubated with the monoclonal antibody against SNAP-25.

magnetic beads. Later (1 day) such positive sorted cells were permeabilized with SLO and treated with BoNT/E before lysis of the cells in SDS sample buffer for gel electrophoresis. Total cellular proteins were analysed by Western blots; mutant and endogenously expressed SNAP-25 proteins (which are indistinguishable in size) were detected using a monoclonal antibody against SNAP-25 (Figure 5). There was much more total SNAP-25 immunoreactivity in cells transfected with the SNAP-25 mutants as compared with controls (confirming strong overexpression of the mutant proteins). Despite such overexpression, the intensity of the band of the endogenous toxin-cleaved SNAP-25 was comparable between cells which do or do not express the toxin-resistant SNAP-25. This is taken as evidence that the strongly overexpressed toxin-resistant mutant did not prevent toxin cleavage of the endogenous wild-type SNAP-25. It follows that the Ca^{2+}-stimulated insulin secretion observed in toxin-treated cells following transfection with the mutant SNAP-25a or b indeed reflects participation of these isoforms in the secretory event.

DISCUSSION

We have shown here that messages for both SNAP-25a and b isoforms are expressed in primary rat pancreatic islet cells as well as in the insulin-secreting cell lines HIT and RIN. SNAP-25a seems to be the most prominent isoform in primary pancreatic B and non-B cells. Using RNase-protection assays, Bark et al. [30] have shown that in contrast with adult brain cortex and spinal cord, where SNAP-25b is the most abundant isoform, in adult adrenal and pituitary cells mainly SNAP-25a is found. Together these data suggest that neuroendocrine cells may express predominantly SNAP-25a.

It has been hypothesized that the different spacing of cysteine residues in the two isoforms as well as a charge difference might influence palmitoylation and hence membrane association and localization [24,42]. This hypothesis is supported by the observation that SNAP-25 immunoreactivity in the nervous system shifts during development from axons and cell bodies to synaptic terminals [43], coinciding with the SNAP-25a isoform being predominant during early development and SNAP-25b being the major isoform in the adult [30,31]. Furthermore, in nerve-growth-

factor-differentiated PC12 cells it has been shown that SNAP-25a is located in the cell body and along axons, whereas SNAP-25b is targeted to the terminals of cellular processes [30]. Using immunofluorescence staining we have observed previously that SNAP-25 is present not only at the cell membrane but also in an intracellular perinuclear region in pancreatic B cells [39]. It is therefore possible that one isoform is localized specifically at the plasma membrane and that the other is in the perinuclear region. Our results in HIT cells show, however, that the cellular localization of the Myc-tagged SNAP-25 isoforms corresponds to that seen for the endogenous SNAP-25. They localize to the entire plasma membrane and in some cells to a perinuclear region. We have been using SNAP-25 Myc-tagged at the N- or C-terminal sides with similar results, suggesting that tagging does not perturb targeting of the two isoforms. Overexpression could have saturated the cellular targeting machinery, but even after careful examination of cells expressing either high or very low levels of the Myc-tagged isoforms (following transient transfection) we did not observe any significant difference.

SNAP-25 is thought to be implicated in two different fusion events of vesicles with the plasma membrane. First, several assay systems have shown that SNAP-25 is essential for the fusion of synaptic vesicles and dense-core granules with the plasma membrane [39,44–51]. Second, SNAP-25 may be involved in membrane expansion and thus fusion of plasma membrane precursor vesicles with the plasma membrane, because cleavage of SNAP-25 by BoNT or reduction of SNAP-25 expression after antisense-oligonucleotide treatment prevents neurite elongation [49,52]. The developmental regulation of the two isoforms, the differential cellular localization in PC12 cells and the fact that SNAP-25a is up-regulated during sprouting of neuronal cells after nerve injury [31] has lead to the suggestion that SNAP-25a may be predominantly involved in the fusion of plasma-membrane precursor vesicles with the plasma membrane, whereas SNAP-25b would be implicated in the fusion of synaptic vesicles at the synapse [42,53]. So far no tools are available to test this hypothesis. Here, we describe the production of toxin-resistant SNAP-25 mutants, which allowed us to test for a possible functional difference between the two isoforms in insulin secretion. In our assay system both isoforms were able to reconstitute insulin secretion from toxin-treated cells even when their expression level was comparable with that of endogenous SNAP-25. This is in contrast with SNAP-23, which was also able to reconstitute insulin secretion in this assay, but only when highly overexpressed [40], indicating that SNAP-25 is more efficient in this fusion event than SNAP-23. We were thus able to detect a functional difference between SNAP-25 and SNAP-23, but not between the SNAP-25a and b isoforms. The existence of two SNAP-25 isoforms in insulin-secreting cells may therefore simply represent redundancy. Alternatively, it may be responsible for the fine tuning of the secretory event, which we may not be able to detect in our assay system, or it may serve a completely different function.

In conclusion, messages for both SNAP-25 isoforms are expressed in insulin-secreting cells and the two proteins appear to be targeted to the plasma membrane as well as to a perinuclear region. Under our assay conditions, both SNAP-25 isoforms are able to function in the process of insulin secretion with comparable efficiency. This is in contrast with what might have been expected according to the hypothesis that in neurons SNAP-25a may be implicated in membrane expansion and SNAP-25b in synaptic transmission. The toxin-resistant mutants described here can now be used in other assay systems and cell types in order to compare directly the function of the two SNAP-25 isoforms. By introducing further mutations into these mutants it will also be possible to screen different domains of SNAP-25 or post-translational modifications for their functional importance in regulated secretion.

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